Links between bacteria derived from penguin guts and deposited guano and the surrounding soil microbiota

Wen Chyin Yew [1, 2, 3] • David Anthony Pearce [1, 4, 5] • Michael James Dunn [4] • Stacey Adlard [4] • Siti Aisyah Alias [1, 3] • Azizan Abu Samah [1] • Peter Convey [1, 4]

[1] National Antarctic Research Centre, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[3] Institute of Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
[4] British Antarctic Survey, NERC, High Cross, Madingley Road, Cambridge CB3 0ET, United Kingdom
[5] Department of Applied Sciences, Faculty of Health and Life Sciences, University of Northumbria at Newcastle, Ellison Building, Northumberland Road, Newcastle upon Tyne, Tyne and Wear NE1 8ST, United Kingdom

Corresponding Author:
Wen Chyin Yew
Email address: wen.c.yew@gmail.com
Telephone: +603-79674634
Fax: +603-79605935

Abstract:

Penguins are an important indicator of marine ecosystem health and a major contributor of nutrients to terrestrial ecosystems in Antarctica. Their stomach microbiota is influenced by both the prey consumed and their foraging environment in the sea. As penguins feed at sea and breed on land, they might be expected to transfer microbes (e.g. prey-associated and marine bacteria) as well as nutrients from their stomachs while regurgitating food or in their guano to the surrounding terrestrial environment. However, most research attention to date has focused separately on the penguin gut microbiota (via cloacal/guano samples) and the terrestrial soil microbiota, and any relationship between them has yet to be established. Here we analysed the bacterial communities in stomach regurgitates and cloacal swabs from the same individual birds, freshly deposited guano and rookery soils of two *Pygoscelis* penguins that breed sympatrically on Signy Island (South Orkney Islands, maritime Antarctic) using a high-throughput DNA sequencing method. Our data do not support the hypothesis that bacteria transferred from penguin guts and/or deposited guano make a significant contribution to the communities of the surrounding terrestrial microbial ecosystem. In both penguin species, composition of bacterial communities differed between the four sample types, with Jaccard similarities ranging between 10 and 36%. Assemblages of

the dominant and co-occurring bacterial communities in rookery soils were either significantly negatively correlated or not correlated with the three other sample types. Sample-specific communities were also identified in this study, contributing around 63% of the identified diversity overall.

Keywords: Antarctic • Bacterial input • Microbial diversity • Terrestrial environment

Introduction:

Penguins are an important indicator species of marine ecosystem health in Antarctica, being amongst the top marine consumers (Brooke 2004), with populations strongly influenced by environmental factors (Boersma et al. 2009; Forcada and Trathan 2009; Boersma and Rebstock 2014). In our recent study (Yew et al. 2017), prey-associated and marine bacteria were confirmed to be present in the microbial communities of penguin stomach regurgitates, suggesting an influence of their diet and foraging environment on the gut microbial community.

Penguins are also a key contributor of nutrients to terrestrial ecosystems in Antarctica (Heine and Speir 1989). Guano deposited by penguins fertilises the typically nutrient-poor Antarctic soils by providing the organic materials, such as nitrogen, organic carbon and phosphorus, required to start the development of ornithogenic soils in and around their rookeries (Heine and Speir 1989; Hofstee et al. 2006; Kim et al. 2012; Ball et al. 2015). These nutrient-rich ornithogenic soils are often linked with the succession of a variety of bacteria (Aislabie et al. 2009; Chong et al. 2009; Kim et al. 2012; Ma et al. 2013; Wang et al. 2015) and other microflora (Vidal et al. 2003; Bokhorst et al. 2007; Zwolicki et al. 2015) and microfauna (Raymond et al. 2013; Bokhorst and Convey 2016) in Antarctica. In studies of other bird species, the nest environment has been reported to have significant effects on the success of egg incubation and the gut microbiota of nestlings (Lucas and Heeb 2005; Brandl et al. 2014). The ornithogenic soil environment potentially in turn may also impact the development of the gut microbial community in penguin chicks.

Previously, avian faecal indicator bacteria, such as *Escherichia coli*, *Enterococcus* spp. and *Enterobacter* spp., have been identified in bird-impacted soil (Trawińska et al. 2016), sand (Whitman and Nevers 2003; Staley et al. 2016) and aquatic (Jiang et al. 2007) environments. As penguins feed in the sea and breed on land, the growth of certain bacteria (e.g. prey-associated and marine bacteria) in the stomach may have an influence on penguin gut microbiomes, and further subsequently be input to the soil microbiota either through defecation or during regurgitation of food to chicks. However, although the establishment of gut microbes in the faeces and surrounding soils has been reported previously in mammals (Faedo et al. 2002) and in termites (Otani et al. 2016), no similar reports appear to exist for bird species including penguins, with studies being limited to the interactions between bird gut microbiota and the nest environment (Hird et al. 2014; Goodenough et al. 2017).

In penguins, researchers have used studies of deposited guano or cloacal swabs to make inferences about gut microbiota (Zdanowski et al. 2004; Banks et al. 2009; Dewar et al. 2013, 2014; Barbosa et al. 2016), and also on the correlations between soil physio-chemical properties and microbiota in rookery soils, in order to study potential microbial roles in ornithogenic soil nutrient cycling (Chong et al. 2009; Kim et al. 2012; Ma et al. 2013). But research is yet to address the converse process of transfer of penguin internal gut microbes into their deposited faecal material and hence potentially into the surrounding terrestrial microbial community. Understanding the role of any link between penguin gut microbes and those of the surrounding terrestrial ecosystem is important as there could be multiple impacts, including on the soil nutrient cycling-associated microbes, the penguin chicks, and the surrounding free-living microfloral and microfaunal communities.

The objectives of this study were 1) to compare the bacterial community compositions between stomach regurgitates, cloacal swabs, freshly deposited guano and rookery soils of two *Pygoscelis* penguin species, 2) to correlate assemblage patterns of the dominant and the co-occurring bacterial communities between the four sample types, and 3) to characterise whether and if so which gut/guano bacterial community members are input to the surrounding soils. As penguins clearly input biogenic material to the surrounding terrestrial environment through regurgitation and defecation, we hypothesised that correlations between the bacterial communities across the four sample types would be present.

Materials and methods:

Sample collection and DNA extraction

This study was conducted at the Gourlay Peninsula (60°43.586’ S, 45°35.063’ W) breeding colony of Adélie (*Pygoscelis adeliae*) and Chinstrap (*Pygoscelis antarctica*) Penguins on Signy Island, South Orkney Islands (Fig. 1) during the 2013/14 breeding season. A total of six stomach regurgitates, six cloacal swabs, six guano and six rookery soil samples were collected from the two *Pygoscelis* penguins (n = 3 for each penguin species) into sterile tubes using tools that were cleaned with 70% ethanol. Penguin stomach regurgitates and cloacal swabs were sampled at the shore close to the breeding bird colonies (i.e. the main route of penguin travel between the sea and the nearby colonies) (Lynnes et al. 2004). Regurgitated ingesta samples of penguins returned from the sea were obtained by stomach flushing (Wilson 1984) following CEMP Standard Methods (CCAMLR 2003), as part of the standard sampling protocol of the long-term monitoring programme of the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) Ecosystem Monitoring Programme (CEMP) on Signy Island. On the spot, cloacal swabs were sampled using sterile swabs from the birds whose stomachs had been flushed for the stomach regurgitate sampling, whilst freshly deposited guano of the breeding birds were collected within the nearby colonies. On the same day or not later than a week depending on weather and logistic constraints, rookery soils (2-5 cm depth from the soil surface) were collected within the same colonies of the breeding birds. As soon as possible after returning to the laboratory at the

British Antarctic Survey's Signy Island research station (1-3 h after collection), total DNA was extracted from the stomach regurgitates using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) (Yew et al. 2017), and from the cloacal swabs, guano and rookery soil samples using the Power Soil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, USA), following the manufacturers' instructions.

16S V4 gene fragment amplification, sequencing and quality filtering

We employed a high-throughput sequencing approach (Illumina MiSeq) to access the bacterial community composition in the samples studied based on the variable region 4 (V4) of the bacterial 16S rDNA. The use of this high-throughput sequencing approach enabled us to produce a high resolution profile of bacterial community composition (Caporaso et al. 2011; Suenaga 2012) for each sample type, and hence allowed a robust comparative analysis across the samples studied. The V4 region of the 16S rRNA gene, which was widely used to study bacterial and archaeal communities in the Earth Microbiome Project, was amplified using the adapted PCR primers (F515 and R806) and the polymerase chain reaction (PCR) as described by Caporaso et al. (2011). DNA quality was checked using a NanoDrop 2000c (Thermo Scientific, Waltham, Massachusetts, USA) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA). DNA libraries were prepared and performed in the MiSeq system for paired-end runs following the manufacturer's instructions (Illumina Inc., San Diego, California, USA). The generated sequences were trimmed and demultiplexed using MiSeq Reporter v2.5 (Illumina Inc., San Diego, California, USA). Forward and reverse reads were merged using the make.contigs command in Mothur with default parameters (Kozich et al. 2013). The generated fasta files were uploaded to an open source metagenomics RAST (MG-RAST) server (Meyer et al. 2008) for quality filtering processes and downstream analyses.

Classification of bacterial community composition in individual samples

Bacterial species-level classification based on the 16S rDNA short regions is imperfect, due to insufficient separation of closely related species (Janda and Abbott 2007; Tindall et al. 2010), even though a commonly accepted 97% sequence similarity threshold has been used elsewhere (Faith et al. 2013; Nakayama et al. 2013). However, the aim of this study was simply to generate composition profiles of bacteria communities that were present in the samples studied to enable comparison between different sample types, rather than provide a detailed taxonomic assessment of surviving or growing bacteria in each sample. In this study, bacterial community compositions of penguin stomach regurgitates, cloacal swabs, guano and rookery soils were classified at an operational taxonomic unit (OTU) level, with a minimum sequence identity threshold of 99%. OTUs were identified using the BLAST-like alignment tool (BLAT) search to the ribosomal database project (RDP) classifier implemented in the MG-RAST server, with maximum e-value set to 10^{-5} and minimum alignment length set to 50 nucleotides (Meyer et al. 2008). In order to limit the impact of sequencing errors, OTUs represented by less than three reads (singletons and doubletons) were removed following Andersson et al. (2008) and Lazarevic et al. (2009), and were not considered further.

Comparative analyses of bacterial communities across the four sample types

To visualise the overall bacterial community relationships across all 24 individual samples studied, principal coordinate analysis (PCoA) was performed based on the Bray-Curtis distance on normalised OTU annotation data (Meyer et al. 2008).

To test whether there were any significant differences in the mean values of OTU richness among the four types of samples (stomach regurgitates, cloacal swabs, guano and rookery soils) in Adélie and Chinstrap Penguins, the total OTUs annotated (with 3 or more reads) for all samples were checked for normal distribution, prior to one-way analysis of variance (ANOVA) with a *post hoc* comparison using Tukey's honestly significant difference (HSD) test (IBM SPSS Windows version 19.0, Armonk, New York, USA).

As Illumina MiSeq is a semi-quantitative method (Hirsch et al. 2010), the bacterial community composition in Adélie and Chinstrap Penguin samples was analysed based on presence/absence data of the annotated OTUs. The Jaccard index was used to calculate the percentage of bacterial composition similarity between any two sample types across the four types of samples obtained. A Venn diagram was generated using a web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to show the proportions of unique and shared OTUs between penguin stomach regurgitates, cloacal swabs, guano and rookery soils.

To examine assemblage patterns of the dominant and the co-occurring bacterial communities between the four sample types, Spearman rank multiple correlation analysis was conducted on the relative abundance of the frequently encountered OTUs (≥0.1%) and the shared OTUs. Any two sample types were considered positively correlated when a statistically significant ($p < 0.05$) Spearman coefficient (r_s) > 0.19 was obtained (Fowler et al. 1998).

Nucleotide sequence accession numbers

Sequences were deposited in MG-RAST (Meyer et al. 2008) with accession numbers listed in Table 1.

Results:

OTU classification and sample coverage

A total of 1,381 and 1,261 bacterial species-level OTUs were annotated from Adélie and Chinstrap Penguins, respectively, with individual samples ranging from 31 to 337 OTUs in Adélie Penguins, and from 31 to 243 in Chinstrap Penguins (Table 1). Besides unclassified bacteria, the annotated OTUs from Adélie Penguins were closely matched to a total of 260 bacterial genera belonging to 17 bacterial phyla. In Chinstrap Penguins, a total of 247 bacterial genera were assigned, also belonging to 17 phyla. Following the rare-biosphere threshold proposed by Lynch and Neufeld (2015), approximately 24.2% (6-70 OTUs per sample) of these OTUs in Adélie Penguins, and 32.7% (8-72 OTUs per sample) in Chinstrap

Penguins accounted for ≥0.1% of relative abundance, and were considered as the dominant community members. OTUs accounting for <0.1% were considered representative of rare community members. The complete lists of annotated OTUs in individual samples of Adélie and Chinstrap Penguins, along with the abundance of each OTU, are provided in Online Resources 1 and 2.

Good's coverage (Good 1953) showed that the sampling completeness averaged 99.8% (ranging from 97.8 to 100.0%) (Table 1), confirming that the coverage for all samples studied was sufficient to permit comparative bacterial community analysis.

Overall bacterial community relationships

Across all 24 samples obtained from the two penguin species, PCoA (Fig. 2) revealed closer bacterial community relationships between stomach regurgitates and cloacal swabs, and between cloacal swabs and guano. However, lower bacterial community relationships were apparent between rookery soils and the three other sample types, particularly the stomach regurgitates.

Comparative analyses between the four sample types in Adélie Penguins

In Adélie Penguins, significant differences were observed in the OTU richness between the four sample types studied (one-way ANOVA, $F(3,8) = 5.320$, $p = 0.026$). *Post hoc* comparisons with Tukey's HSD (Table 2) showed that the mean value of OTU richness of rookery soils ($X \pm SE = 230 \pm 66$ OTUs, $n = 3$) was significantly higher ($p < 0.05$) than that of stomach regurgitates ($X \pm SE = 42 \pm 6$ OTUs, $n = 3$) or cloacal swabs ($X \pm SE = 51 \pm 8$ OTUs, $n = 3$), but was not significantly higher than guano ($X \pm SE = 138 \pm 38$ OTUs, $n = 3$). There were no significant differences in the mean values of OTU richness between stomach regurgitates, cloacal swabs and guano.

Excluding unclassified bacteria, paired comparisons of the bacterial community compositions using the Jaccard index (Fig. 3) showed significant differences between the four sample types in Adélie Penguins, with the highest similarity being present between guano and rookery soils (36%) and the lowest similarity between stomach regurgitates and rookery soils (10%). A total of 76, 105, 274 and 384 distinct OTUs were identified in the stomach regurgitates, cloacal swabs, guano and rookery soils, respectively (Fig. 3). Of the 532 distinct OTUs identified, 4.3% were the shared OTUs between the four sample types. Approximately 13% of these shared OTUs were frequently encountered in all four sample types, belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes. The unique OTUs (62% in total) present in stomach regurgitates, cloacal swabs, guano or rookery soils accounted for 3.9%, 4.1%, 16.4% and 37.0%, respectively. About 9.5% of the unique OTUs in stomach regurgitates, 4.5% in cloacal swabs, 6.9% in guano, and 10.2% in rookery soils, belonged to the frequently encountered classification. The unique and the shared OTUs are listed in Online Resource 3.

Spearman rank multiple correlation analysis (Table 3) identified a significant positive correlation in assemblage patterns of the frequently encountered OTUs between Adélie

Penguin stomach regurgitates and cloacal swabs (r_s = 0.297, n = 122, p = 0.001), but a significant negative correlation between stomach regurgitates and rookery soils (r_s = -0.275, n = 122, p = 0.002). No significant correlations were observed between the frequently encountered OTU assemblages of stomach regurgitates and cloacal swabs with guano or rookery soils. In addition, clear differences were apparent at bacterial phylum level (Fig. 4), where a shift in assemblage patterns appeared in the dominant phyla. Community members whose representation increased between stomach regurgitates and rookery soils included Actinobacteria (<0.1 to 1.6%), Bacteroidetes (0.6 to 35.0%), Proteobacteria (5.6 to 11.1%) and unclassified bacteria (0.8 to 28.2%). Firmicutes (65.6 to 22.9%) and Tenericutes (27.0 to 0.1%), in contrast, showed a decrease between stomach regurgitates and rookery soils.

In the assemblage patterns of shared OTUs (Table 3), a significant positive correlation was found between Adélie Penguin stomach regurgitates and cloacal swabs (r_s = 0.445, n = 23, p = 0.033). Stomach regurgitates and cloacal swabs showed no significant correlations with guano or rookery soils. Amongst the co-occurring community members (Fig. 5), those which increased in occurrence between stomach regurgitates and rookery soils were closely related to *Psychrobacter* (1.1 to 6.1%) and *Tissierella* (<0.1 to 11.0%). OTUs assigned to *Clostridium* (61.7 to 2.5%) and *Mycoplasma* (30.8 to <0.1%) decreased in frequency between stomach regurgitates and rookery soils. OTUs assigned to *Helicobacter* were relatively abundant in cloacal swabs and guano but not in stomach regurgitates or rookery soils.

Comparative analyses between the four sample types in Chinstrap Penguins

In Chinstrap Penguins, again there were significant differences in the OTU richness between the four sample types studied (one-way ANOVA, $F(3,8) = 7.317$, p = 0.011). *Post hoc* comparisons with Tukey's HSD comparison (Table 2) indicated that the mean value of OTU richness of rookery soils ($X \pm$ SE = 202 ± 23 OTUs, n = 3) was significantly higher ($p <$ 0.05) than those of stomach regurgitates ($X \pm$ SE = 56 ± 3 OTUs, n = 3), cloacal swabs ($X \pm$ SE = 80 ± 41 OTUs, n = 3) and guano ($X \pm$ SE = 83 ± 11 OTUs, n = 3). There were no significant differences in the mean values of OTU richness between stomach regurgitates, cloacal swabs and guano.

Excluding unclassified bacteria, Chinstrap Penguins harboured significantly different community compositions in the four sample types. The Jaccard index (Fig. 3) showed the highest similarity was present between cloacal swabs and guano (28%) and the lowest similarity was between stomach regurgitates and rookery soils (13%). A total of 106, 184, 154 and 325 distinct OTUs were identified in the stomach regurgitates, cloacal swabs, guano and rookery soils, respectively (Fig. 3). Approximately 5.3% of the 490 distinct OTUs identified were shared between the four sample types, and 7.7% of these shared OTUs were frequently encountered in all four sample types. Again, these included community members of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes. The unique OTUs (64% in total) present in stomach regurgitates, cloacal swabs, guano or rookery soils accounted for 5.7%, 13.5%, 6.5% and 38.0%, respectively. About 25% of the unique OTUs in stomach regurgitates, 12.1% in cloacal swabs, 15.6% in guano, and 5.9% in

rookery soils were frequently encountered. The unique and the shared OTUs are listed in Online Resource 4.

Spearman rank multiple correlation analysis (Table 3) identified significant negative correlations in assemblage patterns of the frequently encountered OTUs between Chinstrap Penguin rookery soils and stomach regurgitates (r_s = -0.434, n = 119, p < 0.0001) or cloacal swabs (r_s = -0.399, n = 119, p < 0.0001). No significant correlations were detected in the frequently encountered OTU assemblages between stomach regurgitates, cloacal swabs and guano. Community members whose representation increased between stomach regurgitates and rookery soils included the phyla Actinobacteria (<0.1 to 1.7%), Bacteroidetes (0.7 to 56.3%) and unclassified bacteria (10.5 to 17.6%) (Fig. 4). Proteobacteria (20.2 to 14.9%) and Tenericutes (58.5 to <0.1%) showed a decrease between stomach regurgitates and rookery soils.

In the assemblage patterns of shared OTUs (Table 3), Chinstrap Penguin cloacal swabs showed a significant positive correlation with stomach regurgitates (r_s = 0.497, n = 26, p = 0.010), and a significant negative correlation with rookery soils (r_s = -0.399, n = 26, p = 0.043). No significant correlations were detected between stomach regurgitates and guano or rookery soils. Amongst the co-occurring community members (Fig. 5), OTUs closely related to *Flavobacterium* (<0.1 to 9.1%) and *Tissierella* (<0.1 to 5.6%) showed an increase between Chinstrap Penguin stomach regurgitates and rookery soils. OTUs assigned to *Clostridium* (7.2 to 0.9%) and *Mycoplasma* (67.9 to <0.1%) decreased in occurrence between stomach regurgitates and rookery soils. OTUs assigned to *Fusobacterium* were relatively abundant in cloacal swabs and guano but not in stomach regurgitates or rookery soils.

Discussion:

Both *Pygoscelis* penguins examined here revealed similar results with respect to bacterial communities differed between the four different sample types studied, despite potential inter- and intra-specific variations in the microbiota of samples studied between the two penguin species (Yew et al. 2017). Our data showed that the bacterial communities in penguin stomach regurgitates, cloacal swabs, freshly deposited guano and rookery soils were significantly different in terms of the OTU richness, community composition, and assemblage patterns of the dominant and the co-occurring community members. In addition, while Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes were frequently found in all four sample types, the representation of these phyla shifted across the different sample types. Although interactions between avian gut microbiota and the nest environments have previously been reported (Lucas and Heeb 2005; Goodenough et al. 2017), despite the detection of avian faecal-indicator bacteria (e.g. *Escherichia coli*, *Enterococcus* and *Enterobacter*) in bird-impacted areas (Whitman and Nevers 2003; Jiang et al. 2007; Staley et al. 2016; Trawińska et al. 2016), there appear to be no published comparative analyses between the microbiota of guts, faeces and the habitat soils of birds (including penguins). Previously, in studies of a very different taxonomic group, detailed

comparative investigations between the microbiota of gut, deposited materials and surrounding soils have been reported in termites (Fall et al. 2007; Makonde et al. 2015; Manjula et al. 2016). While different methods were used to generate the data, these studies reported similar findings to those obtained here, with significant differences being observed in the bacterial communities obtained from termite guts, mounds and the surrounding soils. These studies also reported a frequency shift in co-occurring bacterial phyla between termite guts and the surrounding soils. Our main finding is consistent with these comparative investigations on termites, although in detail the bacterial community compositions (particularly in terms of genera) identified in penguin samples were different from those of termites.

Rookery soils of both *Pygoscelis* penguins harboured the richest bacterial communities, followed by guano, cloacal swabs and stomach regurgitates. This is also in accordance with the termite study reported by Makonde et al. (2015), where termite-associated soils had higher bacterial diversity than termite guts. Rookery soil bacterial phyla composition in this study was similar to previously described rookery soils on Signy Island (Chong et al. 2009) and other locations in Antarctica (Kim et al. 2012; Bottos et al. 2014; Rampelotto et al. 2014), with the predominant community members being Actinobacteria, Aquificae, Bacteroidetes, Firmicutes, Fusobacteria, Gemmatimonadetes, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes and Verrucomicrobia. Although 78% of the assigned bacterial phyla in total that were present in stomach regurgitates, cloacal swabs and/or guano were also present in rookery soils, the bacterial OTU composition of rookery soils was clearly different from stomach regurgitates, cloacal swabs and guano (Jaccard similarities ranged between 10 and 36%). The dominant and the co-occurring community assemblages of rookery soils were either significantly negatively correlated or not correlated with the three other sample types. Together, these data suggest that there is very low establishment probability of microbes from penguin internal gut and/or deposited guano into the surrounding terrestrial microbial ecosystem, where these microbes might possibly be constrained by the differences in physicochemical properties and nutrient availabilities between the two distinct environments.

Amongst the co-occurring bacterial community members between rookery soils and of stomach regurgitates, cloacal swabs, and/or deposited guano, about 12% belonged to previously identified groups of Antarctic marine origins (Cormack and Fraile 1990; Zdanowski and Donachie 1993; Bowman et al. 1997; Staley and Gosink 1999; Purdy et al. 2003; Dickinson et al. 2016). These included OTUs closely related to the genera *Aeromonas*, *Arthrobacter*, *Brachybacterium*, *Corynebacterium*, *Cytophaga*, *Desulfobacterium*, *Desulfobulbus*, *Leeuwenhoekiella*, *Marinobacter*, *Oceanimonas*, *Octadecabacter*, *Planococcus*, *Polaribacter*, *Psychrobacter*, *Psychroflexus*, *Sphingobacterium*, *Sphingomonas* and *Xanthomonas*. OTUs annotated to Antarctic fish and krill-associated bacteria (Kelly et al. 1978; Cormack and Fraile 1990; Ward et al. 2009) accounted for 5% of diversity overall, included the genera *Acinetobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Mycoplasma*, *Nocardioides*, *Pseudomonas* and *Staphylococcus*. Approximately 9% of the OTUs were assigned to bird/penguin host-associated bacteria (Soucek and Mushin 1970; Barbosa and

Palacios 2009; Dewar et al. 2013; Trawińska et al. 2016), belonging to the genera *Campylobacter*, *Clostridium*, *Edwardsiella*, *Enterococcus*, *Erysipelothrix*, *Eubacterium*, *Helicobacter*, *Lactobacillus*, *Plesiomonas*, *Sporosarcina*, *Streptobacillus*, *Streptococcus* and *Veillonella*. These findings indicate that, besides penguin host-associated bacteria, the establishment of transferred prey-associated and marine bacteria from penguins to the surrounding soils during regurgitation and defecation does occur, even though the establishment probability is low. As a majority of these bacterial groups have been reported to degrade and/or produce organic (Bowman et al. 1997; Wong et al. 2000; Mancuso Nichols et al. 2005; Andreína Pacheco et al. 2008, Yau et al. 2013) and/or inorganic (Kelly et al. 1978; Zdanowski and Donachie 1993; Bowman et al. 1997; Purdy et al. 2003; Yau et al. 2013) matter, thus they may potentially play a role in nutrient cycles and transfer processes (Schimel and Schaeffer 2012; Cavicchioli 2015) between Antarctic marine and terrestrial environments.

Guano freshly deposited by both *Pygoscelis* penguins had predominant community members including Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes. This is similar to the bacterial phyla composition in previously reported penguin faecal samples (Banks et al. 2009; Dewar et al. 2013, 2014). Besides rookery soils, freshly deposited penguin guano also showed significant differences in bacterial community composition from those of stomach regurgitates (Jaccard similarity = 14 and 26% in Adélie and Chinstrap Penguins, respectively) and cloacal swabs (22 and 28%, respectively). No significant correlations in the dominant and co-occurring community assemblages were observed between deposited guano and stomach regurgitates or cloacal swabs. In a recent study, chicken faecal and cecal microbiota were reported to be qualitatively similar but quantitatively different (Stanley et al. 2015). Although previously, faecal samples have been widely used as an indicator for the microbial study of the gastrointestinal tract of mammals, our data suggest that, in penguins in particular, deposited guano does not provide a suitable proxy for the study of the stomach microbiota. Similar findings have previously been reported in studies of other bird species (Wilkinson et al. 2016) and of humans (Durbán et al. 2011), where it was suggested that faecal microbiota are not closely representative of gut microbiota.

While data generated using MiSeq do not provide a robust quantitative assessment of community composition at specific level (Hirsch et al. 2010), clear shifts in the assemblages of co-occurring bacteria were observed across the sample types. For instance, in both Adélie and Chinstrap Penguins, the co-occurring OTUs that were assigned to *Clostridium* and *Mycoplasma* decreased in proportional representation from stomach regurgitates to cloacal swabs/guano and rookery soils. These bacteria are commonly found in homeothermic animal hosts, such as birds and mammals (Craven et al. 2001; Elfaki et al. 2002; Aldape et al. 2006). Penguin stomachs are warm (38°C) (Thouzeau et al. 2003) while, at Signy Island, daily soil temperatures measured across a number of locations ranged between -8 and 20°C throughout the year (Davey et al. 1992). Previously, Zdanowski et al. (2005) proposed that temperature is the most important physical factor influencing bacterial transformations during penguin guano decomposition. Factors such as the changes of temperature between the sampled

environments might underlie the decrease in co-occurring bacteria across the three other sample types, and limit the establishment of stomach-specific bacteria in the surrounding soils.

In our study, sample-specific bacterial communities accounted for 62 and 64% of the overall identified community members in Adélie and Chinstrap Penguins, respectively. Previously, in a comparative analysis of termite guts and the associated surrounding soils, Manjula et al. (2016) suggested that such differences in microbial community composition and structure maybe due to the roles of different microbes in each environment. The potential functions of distinct bacterial groups identified in penguin guts (Soucek and Mushin 1970; Barbosa and Palacios 2009; Dewar et al. 2013), deposited guano (Banks et al. 2009; Dewar et al. 2014) and rookery soils (Chong et al. 2009; Kim et al. 2012; Bottos et al. 2014; Rampelotto et al. 2014) have been inferred previously. In the current study, the main functions of these sample-specific bacteria remain unknown, as the sequencing approach used does not provide reliable species-level information. Further research is required to isolate these sample-specific bacteria for functional gene studies in order to understand their roles in the environment.

Conclusions:

Our study revealed clear differences in the bacterial communities of stomach regurgitates, cloacal swabs, freshly deposited guano and rookery soils of two *Pygoscelis* penguins that breed sympatrically on Signy Island. More than half the OTUs identified were sample-specific, suggesting that input/survival of microbes from penguin internal gut and/or deposited materials into the surrounding terrestrial microbial ecosystem might not provide a significant *in situ* contribution to terrestrial soil microbial community composition.

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Compliance with Ethical Standards: All procedures involving animals followed internationally recognised CCAMLR CEMP standard methods and were in accordance with the ethical standards of the British Antarctic Survey.

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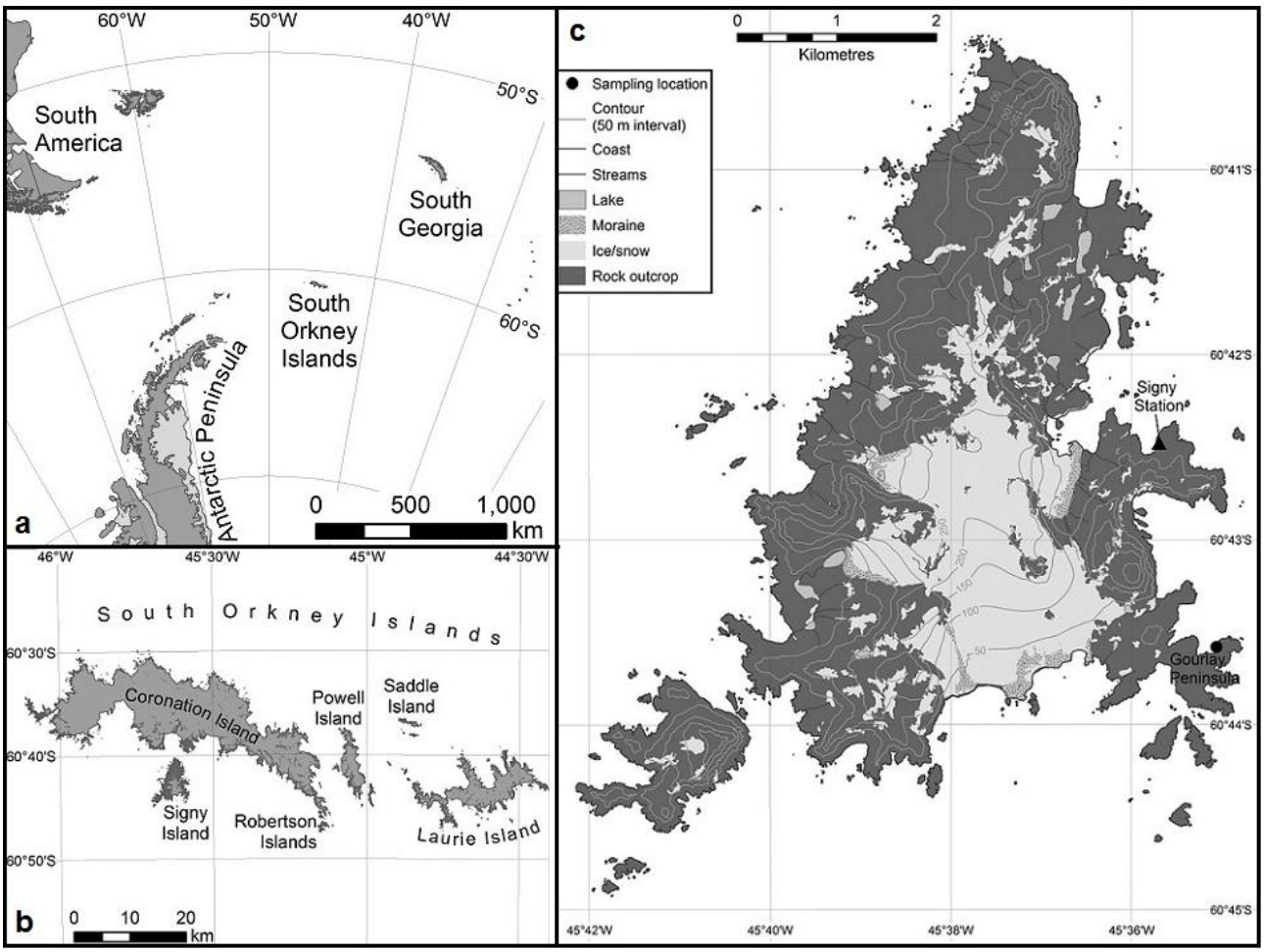

Fig. 1 The locations of a South Orkney Islands in the maritime Antarctic, b Signy Island within the South Orkney Island archipelago, and c Gourlay Peninsula on Signy Island. Map provided by Laura Gerrish, Mapping and Geographic Information Centre, British Antarctic Survey

Table 1 Summary data of individual samples analysed using metagenomics RAST (MG-RAST) server (Meyer et al. 2008)

Accession number	Sample	Sample description	Sampling date	Good's coverage	Total number of OTUs ≥ 3 reads	Total number of OTUs ≥ 0.1%
4604216.3	AS1	Adélie Penguin stomach regurgitate	30/12/13	99.87	31	11
4604111.3	AS2	Adélie Penguin stomach regurgitate	20/1/14	99.54	43	21
4604112.3	AS3	Adélie Penguin stomach regurgitate	20/1/14	99.82	51	18
4605301.3	ACS1	Cloacal swab of AS1	30/12/13	99.97	66	8
4605302.3	ACS2	Cloacal swab of AS2	20/1/14	99.85	40	7
4605303.3	ACS3	Cloacal swab of AS3	20/1/14	99.99	46	6
4705487.3	AG1	Adélie Penguin guano	30/12/13	99.94	100	23
4605306.3	AG2	Adélie Penguin guano	20/1/14	99.98	214	41
4605844.3	AG3	Adélie Penguin guano	20/1/14	99.96	100	16
4705502.3	ARS1	Adélie Penguin rookery soil	6/1/14	99.87	111	54
4605938.3	ARS2	Adélie Penguin rookery soil	20/1/14	99.96	242	70
4606004.3	ARS3	Adélie Penguin rookery soil	20/1/14	99.98	337	69
4606032.3	CS1	Chinstrap Penguin stomach regurgitate	30/1/14	99.91	62	18
4705618.3	CS2	Chinstrap Penguin stomach regurgitate	4/2/14	99.92	51	24
4605300.3	CS3	Chinstrap Penguin stomach regurgitate	17/2/14	99.89	54	18
4605304.3	CCS1	Cloacal swab of CS1	30/1/14	99.96	46	8
4705520.3	CCS2	Cloacal swab of CS2	4/2/14	97.80	31	31
4705435.3	CCS3	Cloacal swab of CS3	17/2/14	99.56	162	72
4606028.3	CG1	Chinstrap Penguin guano	30/1/14	99.92	77	24
4705620.3	CG2	Chinstrap Penguin guano	4/2/14	99.91	69	16
4605937.3	CG3	Chinstrap Penguin guano	17/2/14	99.97	104	23
4705585.3	CRS1	Chinstrap Penguin rookery soil	30/1/14	99.87	165	63
4705549.3	CRS2	Chinstrap Penguin rookery soil	4/2/14	99.90	197	61
4606007.3	CRS3	Chinstrap Penguin rookery soil	19/2/14	99.97	243	54

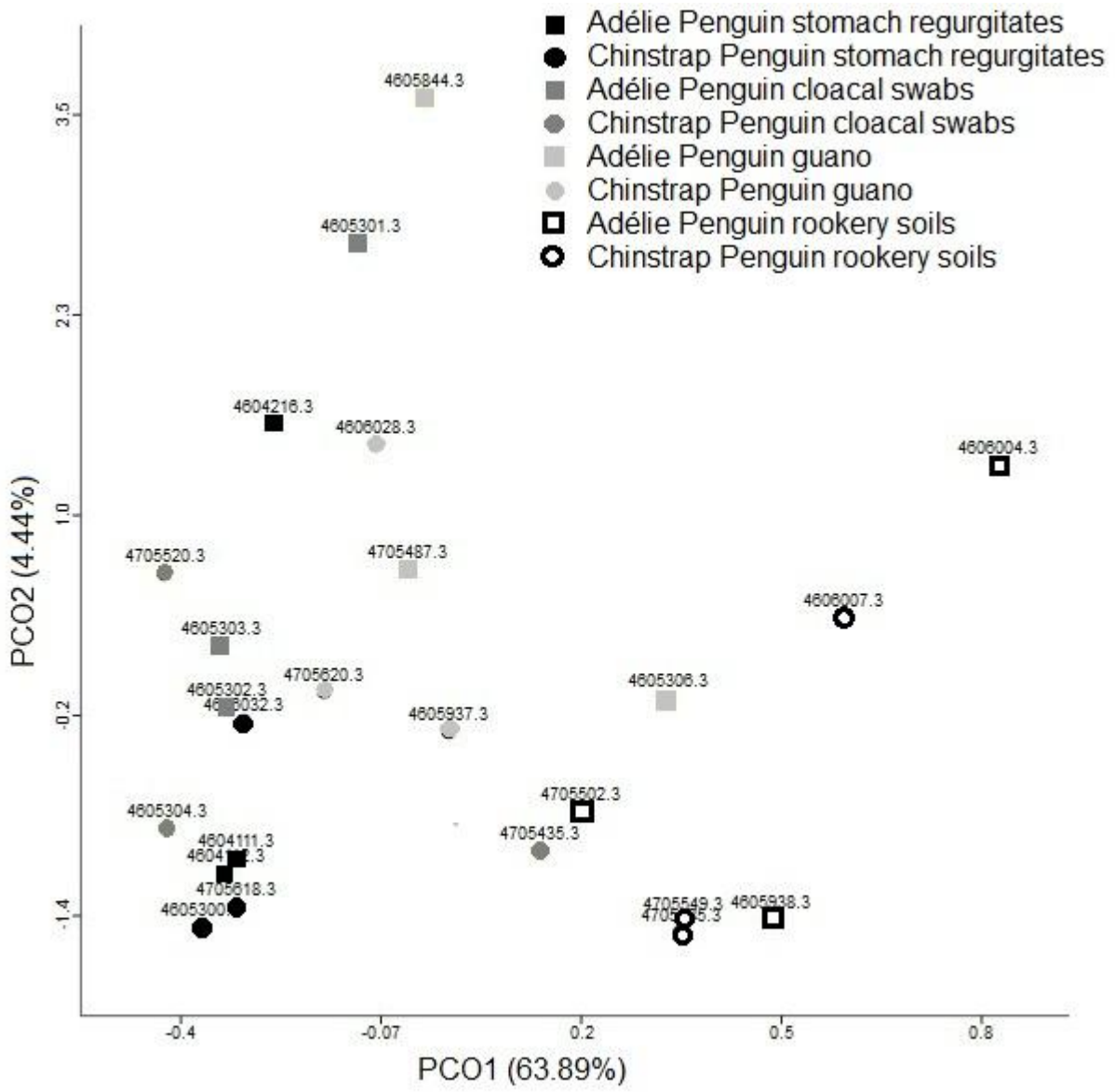

Fig. 2 Principal coordinates analysis (PCoA) of individual samples studied using Bray-Curtis distance on normalised OTU annotation data

Table 2 Mean difference of the OTU richness ($X \pm$ SE) between penguin stomach regurgitates (S), cloacal swabs (CS), guano (G) and rookery soils (RS) analysed by one-way ANOVA with a *post hoc* comparison using Tukey's honestly significant difference test

	Adélie Penguins (n = 3)			Chinstrap Penguins (n = 3)		
	CS	G	RS	CS	G	RS
S	-9 ± 54[a], p = 0.998	-96 ± 54, p = 0.346	-188 ± 54, p = 0.034	-24 ± 34, p = 0.894	-28 ± 34, p = 0.849	-146 ± 34, p = 0.012
CS		-87 ± 54, p = 0.422	-179 ± 54, p = 0.042		-4 ± 34, p > 0.999	-122 ± 34, p = 0.030
G			-92 ± 54, p = 0.381			-118 ± 34, p = 0.035

[a] The mean difference of the OTU richness was calculated by i-j, where i is the sample type indicated in the stub, and j is the sample type indicated in the column.

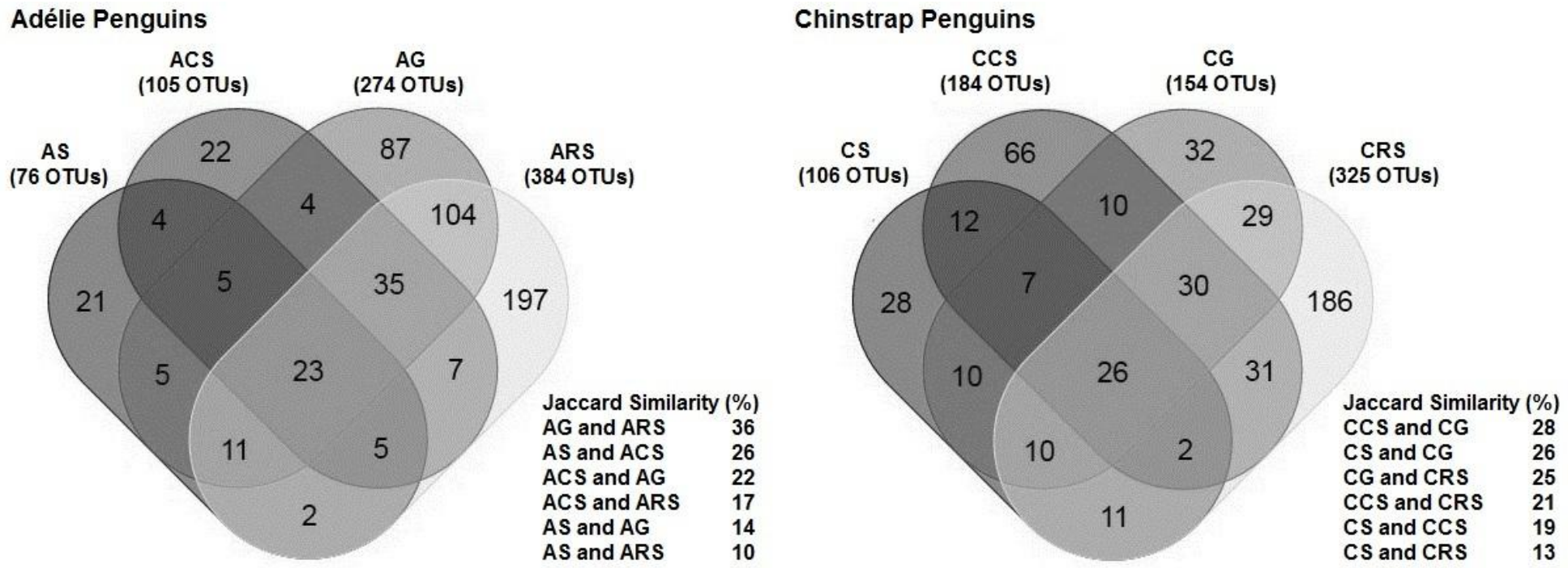

Fig. 3 Venn diagram showed the proportion of unique and shared OTUs between stomach regurgitates (S), cloacal swabs (CS), guano (G) and rookery soils (RS) of Adélie (A) and Chinstrap (C) Penguins. The numbers (in bracket) outside Venn diagram indicate total OTUs found in each type of samples. The numbers inside Venn diagram indicate unique and shared OTUs between the four sample types. Bacterial community composition similarity was calculated using Jaccard index

Table 3 Spearman rank multiple correlation analysis of assemblage patterns of the frequently encountered and the shared operational taxonomic units (OTUs) between stomach regurgitates (S), cloacal swabs (CS), guano (G) and rookery soils (RS) of Adélie (A) and Chinstrap (C) Penguins

Adélie Penguins	Frequently encountered OTUs (n = 122)			Shared OTUs (n = 23)		
	ACS	AG	ARS	ACS	AG	ARS
AS	0.297[a], p = 0.001	0.000, p = 0.996	-0.275, p = 0.002	0.445, p = 0.033	0.131, p = 0.551	-0.214, p = 0.326
ACS		0.149, p = 0.102	-0.086, p = 0.348		0.348, p = 0.104	-0.101, p = 0.645
AG			-0.172, p = 0.059			0.304, p = 0.158
Chinstrap Penguins	Frequently encountered OTUs (n = 119)			Shared OTUs (n = 26)		
	CCS	CG	CRS	CCS	CG	CRS
CS	0.121, p = 0.189	0.039, p = 0.673	-0.434, p < 0.001	0.497, p = 0.010	0.152, p = 0.458	-0.279, p = 0.168
CCS		0.128, p = 0.165	-0.395, p < 0.001		0.242, p = 0.235	-0.399, p = 0.043
CG			-0.082, p = 0.375			-0.302, p = 0.134

[a] Spearman rank multiple correlation coefficient, $r_s > 0.19$ indicated a positive correlation, $r_s < -0.19$ indicated a negative correlation, and $-0.19 < r_s < 0.19$ indicated no correlations (Fowler et al. 1998).

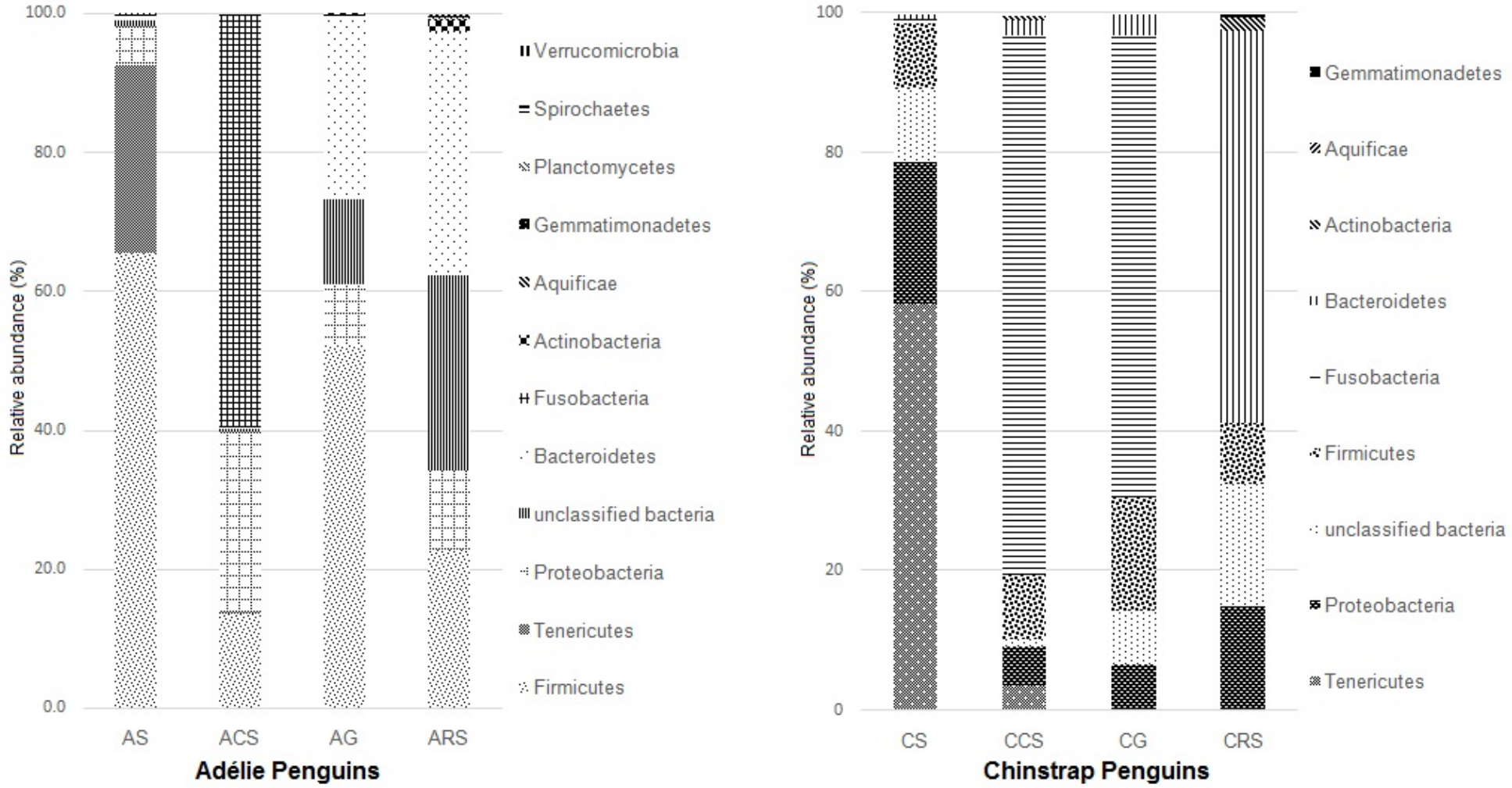

Fig. 4 Assemblage patterns of the frequently encountered (≥0.1%) bacterial phyla in Adélie (A) and Chinstrap (C) Penguin stomach regurgitates (S), cloacal swabs (CS), guano (G) and rookery soils (RS)

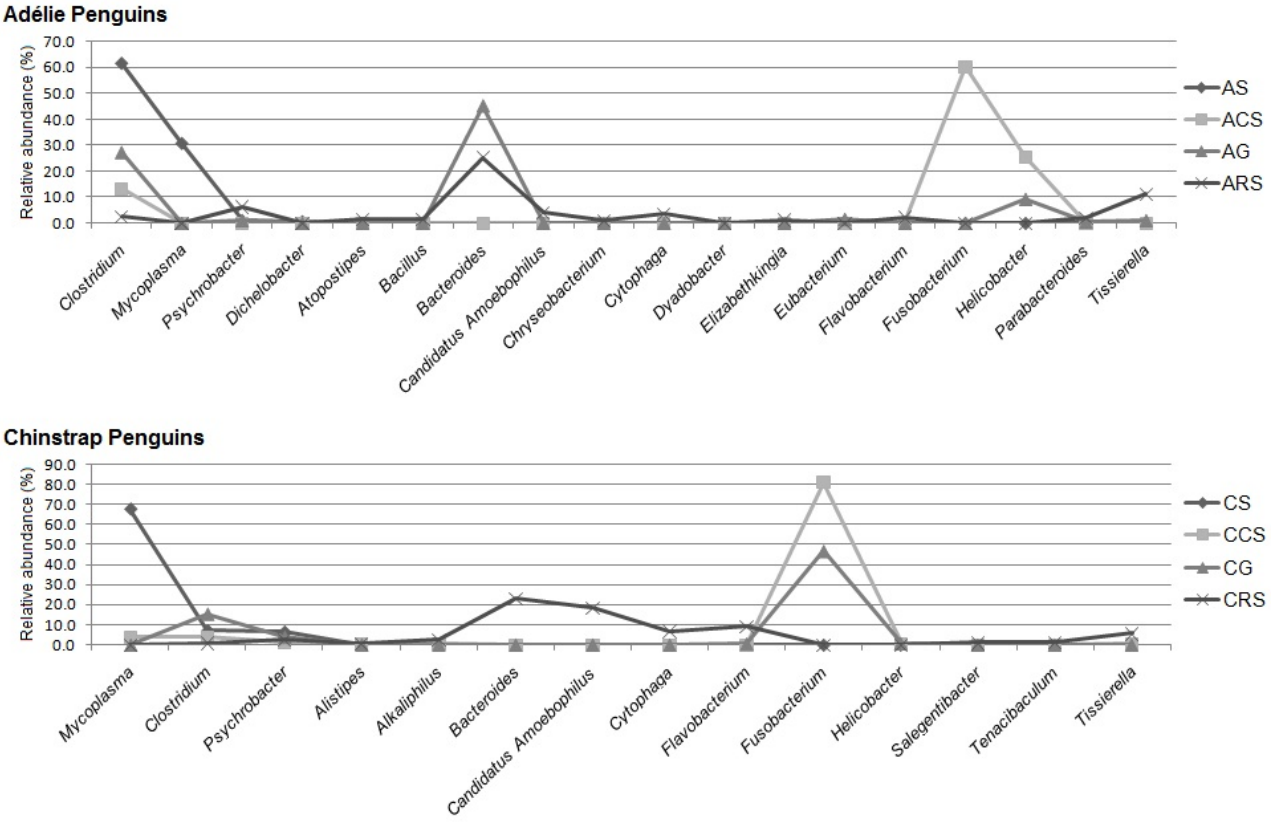

Fig. 5 Assemblage patterns of the co-occurring bacterial genera community members in Adélie (A) and Chinstrap (C) Penguin stomach regurgitates (S), cloacal swabs (CS), guano (G) and rookery soils (RS). Only bacterial genera with relative abundance ≥1% in any sample types are shown